AD_____

Award Number: DAMD17-03-1-0492

TITLE: Endoplasmic Reticulum Stress as a Mediator of Neurotoxin-induced Dopamine Neuron Death

PRINCIPAL INVESTIGATOR: Robert E. Burke, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

REPORT DATE: July 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Annual (1 Jul 2003 - 30 Jun 2004)

4. TITLE AND SUBTITLE
Endoplasmic Reticulum Stress as a Mediator of Neurotoxin-induced Dopamine Neuron Death

5. FUNDING NUMBERS
DAMD17-03-1-0492

6. AUTHOR(S)
Robert E. Burke, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

E-Mail: rb43@columbia.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The molecular processes of programmed cell death (PCD) are important mediators of neural degeneration in Parkinson's disease (PD). The goal of this proposal is to examine in living animals the possible role of ER stress, a mediator of PCD, in dopamine neuron death. This is being done by the study of mice with targeted deletions of CHOP, an upstream transcriptional mediator of ER stress-induced apoptosis. We have demonstrated that CHOP is universally expressed in neurotoxin models of parkinsonism. Assessment of the functional significance of CHOP expression by study of CHOP null mice has shown that in the 6OHDA model immature animals do not have diminished apoptosis, but mature ones do. The null mutation does not protect dopamine neurons in SN in mice in the chronic MPTP model. We have assessed these models for other markers of ER stress. We have performed Northern and in situ hybridization analysis of BiP, and RT-PCR/Southern analysis of the XBP-1 splice variant. Neither is upregulated in these models. We thus far conclude that while CHOP is expressed and uniquely plays a functional role in the adult 6OHDA model, whether it is a participant in a complete ER stress response remains to be determined.

14. SUBJECT TERMS
apoptosis, programmed cell death, 6-hydroxydopamine, MPTP, Parkinson disease

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

There is a growing consensus that the molecular processes of programmed cell death (PCD) are important mediators of neural degeneration in Parkinson's disease (PD) and related disorders. However, while important recent advances in PD research have implicated both environmental and genetic factors in the pathogenesis of the disease, it has been unclear how these factors initiate the PCD cascade. The recent advances in our understanding of the genetic basis of PD, related to synuclein mutations which foster protein aggregation, and parkin mutations which result in a loss of functional ability to ubiquitinate difficult-to-fold proteins, have suggested a possible role for endoplasmic reticulum (ER) stress. In addition, it has been shown by analysis of gene expression in neurotoxin models in tissue culture, that ER stress may play a role in the PCD of dopamine neurons (1,2). The goals of this proposal are to examine in living animals whether CHOP, an upstream transcriptional mediator of ER stress-induced apoptosis, and caspase-12, a downstream mediator, play a role in PCD of dopamine neurons in neurotoxin models of parkinsonism. These goals will be achieved by studying mice with null mutations for these mediators. The sensitivity of the null animals to the induction of apoptosis in dopamine neurons will be examined in well-characterized and validated models of parkinsonism: intrastriatal injection of 6-hydroxydopamine in immature and adult mice, and chronic, systemic injection of MPTP in mice.

BODY

In our original proposal, we submitted preliminary data which indicated that ER stress is likely to occur and to be a mediator of programmed cell death in neurotoxin models of parkinsonism. Our colleague and collaborator on this proposal, Dr Lloyd Greene, had shown that the neurotoxin 6-hydroxydopamine (6OHDA) induces the expression of a number of mediators of an ER stress response in PC12 cells: ATF4, CHOP, BiP, phosphorylated PERK and others (1). A similar induction was noted on treatment of the cells with MPTP. He demonstrated that the ER stress response was likely to be mediating cell death in this culture model because sympathetic ganglion neurons derived from mice null for PERK, a mediator of a protective pathway in ER stress, were more sensitive to 6OHDA (1). Very similar findings were reported by Holtz and O'Malley for MN9D cells (2). The critical question which we therefore sought to address is whether ER stress occurs in these neurotoxin models *in vivo*, and if so, whether it plays a role in mediating PCD. We submitted as preliminary data for this proposal immunohistochemical evidence that CHOP is expressed specifically in dopamine neurons of the SN in a model of intra-striatal injection of 6OHDA in immature rats. We showed that the time course of CHOP expression in these neurons in this model paralleled the induction of apoptosis. We also showed that CHOP is expressed in the SNpc in a chronic MPTP model, which induces apoptosis in dopamine neurons (3). We demonstrated that the expression of CHOP in dopamine neurons in association with apoptosis is specific for these neurotoxin models; CHOP expression is not observed in apoptosis associated with natural cell death, or axotomy-induced augmentation of natural cell death.

On the basis of these preliminary observations, we proposed three tasks to delineate the functional roles of CHOP and caspase-12, a downstream mediator of PCD in ER

stress, in dopaminergic neurotoxin-induced PCD in living animal models. We proposed to do this by studying the effects of null mutations for these mediators on dopaminergic cell death induced by 6OHDA and MPTP.

***Task 1.* To determine if CHOP is a mediator of 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

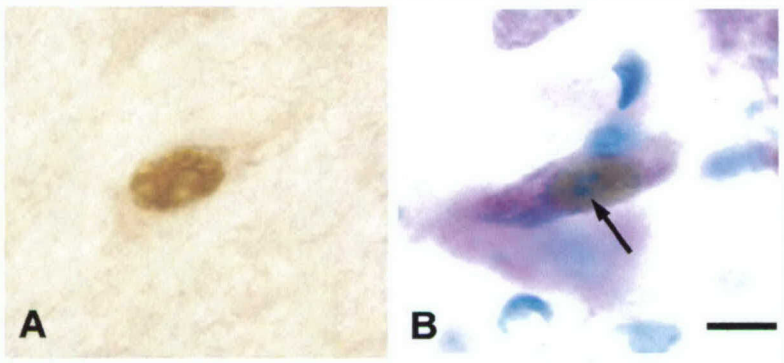


FIGURE 1. Expression of CHOP in SNpc neurons following intra-striatal 6OHDA in adult mice. **A.** Immunoperoxidase labeling of the nucleus of an SNpc neuron at 6 days postlesion. B. Immunoperoxidase labeling and thionin counterstain of an SN neuron at 6 days postlesion. An arrow indicates an apoptotic chromatin clump. Bar = 10 µm.

In this task we proposed to assess the effect of a CHOP null mutation on vulnerability to 6OHDA in both postnatal and adult models of induced apoptotic death in dopamine neurons. Our previous work had only studied induction of CHOP by 6OHDA in postnatal rats. To confirm that it is also induced in mice, and in the adult setting, we performed immunoperoxidase labeling for CHOP in SN tissues following intrastriatal injection of 6OHDA. These experiments did confirm induction of CHOP expression, and they demonstrated co-localization of CHOP labeling with apoptotic chromatin clumps (FIGURE 1).

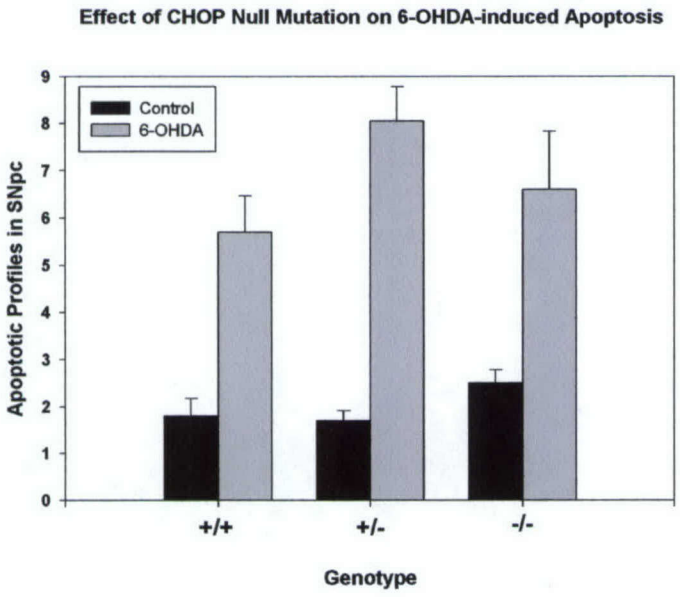


FIGURE 2. The effect of the CHOP null mutation on the induction of apoptosis in dopamine neurons by intra-striatal 6OHDA in postnatal mice. In all three genotypes there is a robust, 3-4 fold induction of apoptosis among dopaminergic neurons, as we have previously reported for postnatal rats (Marti et al, 1997). There was no difference in the homozygous null animals (-/-) as compared to wildtypes or heterozygotes.

As proposed in Task 1, we have assessed the vulnerability of SN dopamine neurons to 6OHDA in CHOP nulls as compared to heterozygote and wildtype littermate control mice in the postnatal model. In this model, postnatal day (PND) 6 mice receive an intrastriatal injection of 6OHDA, and they are sacrificed on post-injection day 4, at the time of the peak of apoptosis induction. In this paradigm, we observed no protective effect of the CHOP null mutation (FIGURE 2).

We have previously demonstrated that induction of apoptosis in postnatal rats by intrastriatal injection of 6OHDA results in two distinct forms of apoptotic death, defined by immunostaining for caspase-3 cleavage products: a form with nuclear staining, which is

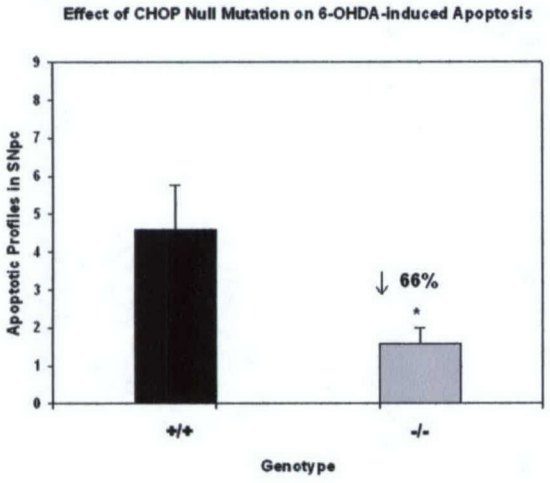


FIGURE 3. The effect of the CHOP null mutation on the induction of apoptosis in dopamine neurons by intra-striatal 6OHDA in adult mice. In the CHOP null adult mice, induction of apoptosis by intra-striatal 6OHDA injection was reduced by 66% (*p<.03, t-test).

the pattern observed in natural cell death and post axotomy; and a form with cytoplasmic staining, which is unique to the 6OHDA model (4). We therefore considered the possibility that some of the induced death in the postnatal model was due to augmented natural cell death (5), which is not mediated by CHOP. In adult mice, this confound would not exist. We therefore examined the vulnerability of SN dopamine neurons to 6OHDA in adult CHOP nulls as compared to wildtype littermate control mice. This study demonstrated that adult CHOP null mice are resistant to the induction of apoptosis by 6OHDA; these animals showed a 66% decrease in the number of apoptotic profiles (FIGURE 3)

Our further plans for this Task now include an additional study of the adult mice, in which we will determine if the CHOP null mutation has a lasting effect on the survival of SN dopamine neurons. Null and wildtype animals will receive intrastriatal injection of 6OHDA; they will be sacrificed at 28 days following injection, and the number of surviving dopamine neurons will be determined by stereology, as originally proposed in Task 1, Part B.

***Task 2.* To determine if ER stress is a general feature of neurotoxin-induced apoptosis in dopamine neurons of the substantia nigra *in vivo*.**

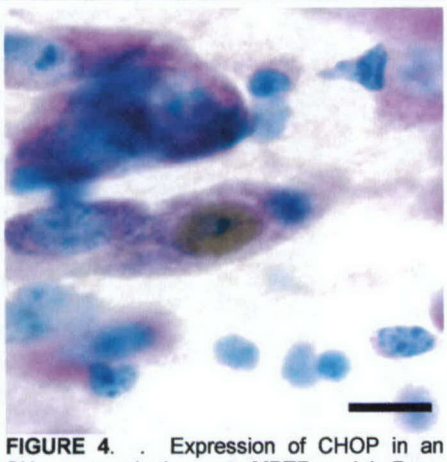
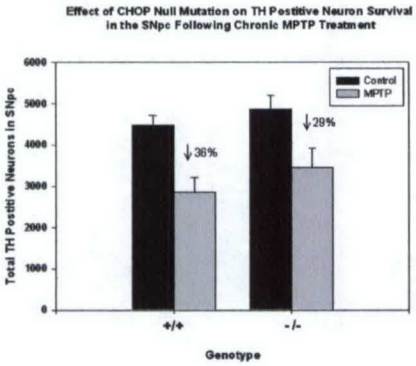


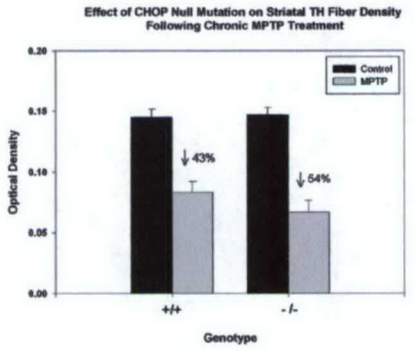
FIGURE 4. . Expression of CHOP in an SNpc neuron in the acute MPTP model. Bar = 10 µm.

In our original proposal, we had planned to determine whether the CHOP null mutation has an effect on vulnerability to MPTP in the model of chronic administration (30 mg/kg/day x 5 days). We had originally proposed to undertake these studies in Year 03, but when our initial observations in the 6OHDA postnatal model were negative, we decided to quickly move on to the MPTP model. In advance of these studies, we decided it would be important to determine whether CHOP expression occurs not only in the chronic MPTP model, as we had shown in our original Preliminary Data, but also in the acute MPTP model (20 mg/kg x 4 doses in one day). These experiments did demonstrate expression of CHOP in the acute MPTP model (FIGURE 4).

We then performed studies as proposed in Task 2: we determined whether the CHOP null mutation had an effect on the vulnerability of SN dopamine neurons to chronic administration of MPTP. We administered MPTP over 5 days, sacrificed the mice at postlesion day 21, and processed the SN tissue for TH immunostaining of dopamine neurons. The number of surviving neurons in null and wildtype animals was determined



A. SN Dopamine neurons



B. Striatal TH density

FIGURE 5. The effect of the CHOP null mutation on dopamine neuron cell body and fiber survival in the chronic MPTP model. **A.** Mice were administered MPTP and then processed for stereologic determination of the number of SN dopamine neurons at 21 days following administration of the final dose. Administration of MPTP resulted in a significant loss of SN dopamine neurons, as previously reported. There was no protective effect of the null mutation. **B.** The same animals assessed in Panel A were also processed for TH immunostaining of dopaminergic fibers in the striatum. The intensity of staining was assessed by optical density measurements. There was no protective effect of the null mutation.

by stereology. These studies were performed in collaboration with Drs Serge Przedborski and Vernice Jackson-Lewis here at Columbia. We found that the null mutation had no effect on the number of surviving dopamine neurons or fibers (FIGURE 5).

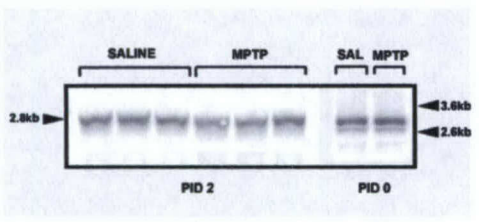
There are two principal explanations for the difference between these results and those obtained in the adult 6OHDA model. It is possible that CHOP plays a role in the 6OHDA model, and not the MPTP model. Such a possibility is suggested by the fact that both Greene and O'Malley found a less robust induction of the ER stress response in the MPTP model as compared to the 6OHDA model (1,2). Alternatively, the difference may be related to the use of two very different read-outs for these studies. In the 6OHDA model, we examined the number of apoptotic profiles in the acute setting. In the MPTP model, we examined long-term survival. In the upcoming year, we will therefore extend these studies to examine the chronic MPTP model in the acute setting, and, as stated above, we will examine the 6OHDA model in the chronic setting.

***Task 3.* To determine if caspase-12 is a mediator of 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

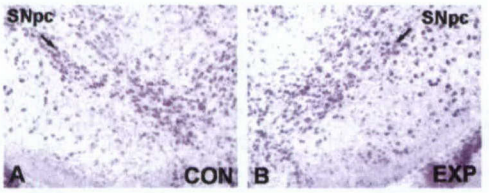
We have the caspase-12 mice in hand, and have expanded the colony, but have not yet started any studies. These studies were originally proposed for year 04.

Studies undertaken in response to Reviewer's comments

Our original proposal received a very fair and thorough review, and we have decided that it is important to address one of the issues raised by one of the Reviewers. It was pointed out that expression of CHOP alone is not definitive evidence for the occurrence of an ER stress response. CHOP induction can occur under circumstances of oxidative stress and amino acid starvation, for example. The Reviewer therefore recommended that we examine other indicators of ER stress in our models. We have selected two. One is the BiP chaperone protein, which is often (but not always) upregulated in ER stress. The other is the ER stress splice variant of the transcription factor XBP-1, which is generally considered to be the most specific indicator of ER stress (personal communications, Drs David Ron; Kazutoshi Mori).



A MPTP (CHRONIC)

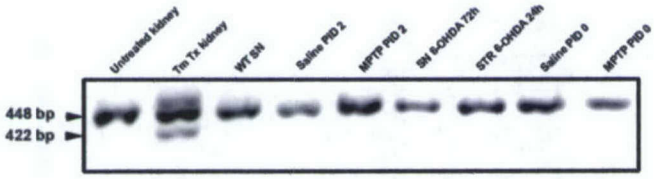


B 6OHDA

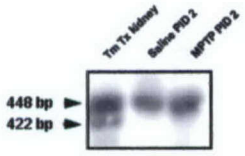
FIGURE 6. Examination of BiP mRNA by Northern and in situ hybridization analysis in the MPTP and 6OHDA models. A. Northern analysis of BiP mRNA in the chronic MPTP model at postinjection days 0 and 2 shows no induction of mRNA expression. B. In situ hybridization analysis of BiP mRNA by non-radioactive in situ hybridization analysis at 48 hours postinjection of 6OHDA in an adult mouse. There is no apparent induction on the Experimental (EXP) side as compared to the Control (CON) side in the SNpc.

We examined the expression of BIP mRNA by Northern analysis in the chronic MPTP model at two time points: post-injection days 0 and 2. At neither time was BiP expression increased (FIGURE 6A). In the adult 6OHDA model, we examined BiP expression by non-radioactive in situ hybridization. No induction was observed at 48 hours postlesion (FIGURE 6B). Therefore there is no induction of this ER stress marker.

We also determined whether the XBP-1 422 bp splice variant could be identified in SN tissues in the acute or chronic MPTP or adult 6OHDA models. For this assessment, we used RNA derived from the kidney of a mouse treated with tunicamycin as a positive control. As shown in FIGURE 7, in the presence of this positive control, the XBP-1 splice variant was not detected in the chronic or acute MPTP models, or the 6OHDA model. There are two possible conclusions. It remains possible that ER stress is occurring in these models, but these markers thereof remain below the limit of detection in these studies conducted at the tissue (as opposed to cellular) level. The second possible conclusion is that CHOP is induced in these models not on the basis of ER stress, but rather some other cellular stress, such as oxidative stress.



A. Chronic MPTP and 6OHDA



B. Acute MPTP

FIGURE 7. RT-PCR/Southern analysis of the 422 bp splice variant of XBP-1 in neurotoxin models of parkinsonism. **A.** The 422 bp splice variant is detected in positive control tissue (tunicamycin-treated kidney (Tm TX), but not in chronic MPTP-treated SN at 0 or 2 days postinjection, or in 6OHDA-treated SN tissue at 72 hours postinjection. B. The 422 bp variant is also not detected in the acute MPTP model.

KEY RESEARCH ACCOMPLISHMENTS

-We have demonstrated that the transcription factor CHOP, a mediator of ER stress-induced apoptosis, is expressed in many of the most important neurotoxin models of parkinsonism: 6OHDA-induced apoptosis in postnatal and mature rats and mice, and MPTP-induced cell death following acute or chronic administration.

-We have demonstrated that CHOP plays a functional role in the adult model of 6OHDA-induced apoptosis. Whether or not it may play such a role in the MPTP model will be determined by further studies planned in Year 02.

-In spite of the expression of CHOP in these models, there is no further evidence at the tissue level for the expression of other markers of ER stress, including BiP and the 422 bp splice variant of the transcription factor XBP-1. It is therefore possible that CHOP expression is due to oxidative stress rather than ER stress in these models.

REPORTABLE OUTCOMES

These results were presented in part at the Annual Meeting of the Society for Neuroscience 2003 (see Appendix):

Silva RS, Oo TF, Jackson-Lewis VJ, Ryu E, Ron D, Przedborski S, Greene LA, Burke RE. The dopaminergic neurotoxins 6-hydroxydopamine (6-OHDA) and MPTP induce expression of CHOP (GADD153) in substantia nigra (SN) in vivo. Society for Neuroscience, 2003.

NOTE: At the time of submission of the Abstract (March 2003) the funding under this award had not begun, so acknowledgement of funding received under this award was not possible on the Abstract submission form. However, **at the time of presentation funding through DAMD17-03-1-0492 was acknowledged.**

CONCLUSIONS

Based on our studies thus far, we firmly conclude that CHOP, a mediator of apoptosis due to ER stress, is upregulated in virtually all of the major neurotoxin models of parkinsonism. Our evidence to date indicates that CHOP is likely to be a functional mediator of apoptosis in the 6OHDA-induced model of parkinsonism. We do not at this time know if genetic ablation of CHOP provides a lasting protection of SN dopamine neurons against 6OHDA-induced apoptosis, or only temporary protection. Our next step in the next funding period will be to determine if CHOP null mice show a lasting increase in the number of surviving SN dopamine neurons in the 6OHDA model. Our current results in the MPTP model suggest that CHOP may not be a mediator of cell death in this model, in spite of the fact that it is robustly upregulated. However, alternatively, it is possible that the CHOP null mutation may delay death in the MPTP model, but not provide lasting protection, as may yet prove to be the case in the 6OHDA model. This possibility will be addressed in the next funding period by acute studies in the chronic MPTP model.

We have found that two important markers of ER stress, the BiP chaperone protein, and the 422 bp splice variant of the transcription factor XBP-1, are not upregulated in the adult 6OHDA model, or the chronic or acute MPTP models. It is therefore possible that the upregulation of CHOP in these neurotoxin models is not mediated by ER stress, but rather another form of cellular stress, such as oxidative injury, which has been postulated to occur in both the 6OHDA and the MPTP models.

REFERENCES

1. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci 2002;**22:**10690-10698.

2. Holtz WA, O'Malley KL. Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. J Biol Chem 2003;**278:**19367-19377.

3. Tatton NA, Kish SJ. In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange. Neuroscience 1997;**77:**1037-1048.

4. Oo TF, Siman R, Burke RE. Distinct nuclear and cytoplasmic localization of caspase cleavage products in two models of induced apoptotic death in dopamine neurons of the substantia nigra. Exp Neurol 2002;**175:**1-9.

5. Marti MJ, James CJ, Oo TF, Kelly WJ, Burke RE. Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of the substantia nigra. J Neurosci 1997;**17:**2030-2039.

Program Number: 204.14 **Day / Time:** Sunday, Nov. 9, 9:00 AM - 10:00 AM

The dopaminergic neurotoxins 6 - hydroxydopamine (6 - OHDA) and MPTP induce expression of chop (GADD153) in substantia nigra (SN) *in vivo*.

R.M.Silva [1*]; T.F.Oo [1]; V.Jackson-Lewis [1]; E.J.Ryu [2]; D.Ron [3]; S.Przedborski [1,2]; L.A.Greene [2]; R.E.Burke [1,2]

1. Dept. Neurol, 2. Dept. Path, Columbia Univ, New York, NY, USA, 3. Dept Cell Biol., NYU, New York, NY, USA

Assessments of patterns of gene expression in vitro in response to induction of cell death by dopaminergic neurotoxins have revealed upregulation of many mediators of the ER stress response, particularly CHOP (GADD153) (Ryu et al., J.Neurosci., 2002; Holtz & O'Malley, JBC, 2003) which has been postulated to mediate apoptosis. However, it has been unknown whether CHOP is likewise induced in vivo. We have therefore assessed the expression of the CHOP protein by immunohistochemistry in animal models in which apoptosis is induced in dopamine (DA) neurons by intrastriatal injection of 6OHDA in postnatal or mature rats or by systemic, chronic injection of MPTP in mice. Intrastriatal injection of 6OHDA in immature rats, which induces apoptosis exclusively, induced CHOP nuclear staining strictly within dopamine neurons of the SNpc, demonstrated by double-labeling for tyrosine-hydroxylase (TH). The time course of CHOP induction paralleled that of induced apoptosis. CHOP induction was not observed in apoptosis due to natural cell death or early target support deprivation by axotomy. CHOP was also induced in SNpc neurons of mature rats following intrastriatal 6OHDA, and in mice following chronic systemic injection of MPTP. To explore the possible functional role of CHOP in the mediation of apoptosis, we have examined the magnitude of induced apoptosis following intrastriatal 6OHDA in postnatal homozygous CHOP null mice in comparison to littermate heterozygote and wild type controls. We find no effect of the null mutation. We conclude that CHOP induction is a universal feature of these neurotoxin models, but its functional role requires further investigation.

Support Contributed By: NS26836, NS38370, PDF, MJFF

Citation: R.M.Silva, T.F.Oo, V.Jackson-Lewis, E.J.Ryu, D.Ron, S.Przedborski, L.A.Greene, R.E.Burke. The dopaminergic neurotoxins 6 - hydroxydopamine (6 - OHDA) and MPTP induce expression of chop (GADD153) in substantia nigra (SN) *in vivo*.. Program No. 204.14. *2003 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience